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Low temperature studies in insects.

Richard M. Duffield
University of Massachusetts Amherst

LOW TEMPERATURE STUDIES IN INSECTS

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RICHARD M. DUFFIELD

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RICHARD M. DUFFIELD

Approved as to style and content by:

T. Michael Peters

Dr. T. Michael Peters
Head of Department and
Chairman of Committee

John H. Nordin

Dr. John Nordin

Lawrence J. Edwards

Dr. Lawrence J. Edwards

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INTRODUCTION

Insects, like all organisms, struggle against their environment. However, insects are considered by many to be the most successful group of living organisms for two main reasons; their prolificacy and their ability to cope with periods of adverse conditions. As a result, insects are found living in a multitude of heterogeneous ecological niches. An interesting aspect of this diverse adaptability is the ability of those distributed in regions having a winter season to survive the sub-zero temperatures. This may be accomplished in any one of the life stages, egg, larvae, pupae, or adult. Therefore it is imperative for the over-wintering state to survive the low temperatures in order to propagate and produce viable offspring.

In general, when ambient temperatures decrease, there is ordinarily a decrease in the physical activity of most insects. In association with this inactivity the organism may exhibit a number of diverse physiological and biochemical responses. As the temperature nears 0^{o} many adult insects will succumb to the low temperature. In contrast, the cold may induce physiological changes causing larvae to pupate, or inducing the onset of diapause. These changes represent an improved physiological state over the previous, for the ecological conditions present. Cool temperatures may initiate the behavioral response seen with the genus Ichneumon (Ichneumonidae, Hymenoptera) in which females seek out quarters for hibernation during the winter. Such responses as these demonstrate the adaptability of insects to changes in the environment.

A most unusual adaptation in some insects at low temperatures is the accumulation of high polyol levels. However, neither the source of polyols, their pathway of synthesis or possible significance of these high levels has been resolved.

This study was conducted to ascertain the relative importance of, and the metabolic pathways by which glycerol is synthesized in certain over-wintering Hymenoptera when they are cold stressed, in hope of understanding how insects over-winter generally. Specifically, the study had two aspects. Initial experiments were designed to give data showing the nature and rate of glycerol production in these insects, whether glycerol was localized in a particular body region, and the effect of time and temperature on both glycerol and glycogen levels.

Another series of experiments involved the injection of ^{14}C-glucose into ants. The initial experiments demonstrated that radioactivity could be detected in the isolated glycerol fraction. Other experiments were designed where, after an initial period of time, ants were sacrificed and extracts fractionated into three major components: glycerol, glycogen, and neutral lipid. It was anticipated that the components would indicate the flux of metabolism taking place with cold stress.

The significance of the processes involved in the tolerance of some insects to sub-zero temperatures is not well understood. However, with the increasing support of the populace for banning insecticide use, other measures of control must be introduced. Thus, more emphasis is being put on the biochemistry of insects, in hope of developing new control procedures.

LITERATURE REVIEW

Until the mid 1950's, much research in cryobiology was concerned with the generalized aspects of the process of acclimation of insects to the cold. However with the advent of new methods of microanalysis, particularly those involving chromatography, a number of previously unsuspected components in insect hemolymph were revealed. One of these components was glycerol, discovered independently by Wyatt and Kalf in the pupal hemolymph of the saturniid moth, Hyalophora cecropia (L.) (Wyatt and Kalf, 1958) and by Chino in the diapausing eggs of the silkworm Bombyx mori (L.) (Chino, 1957). This led to the suggestion by Salt of its possible role in cold hardiness, (Salt, 1961). The existence of high polyhydric alcohol levels provided a basis for a theory of frost resistance.

With the genesis of cryobiology, a number of synonomous terms arose. It would be appropriate to define a standard set of terms in order to eliminate any confusion. Low temperature is a relative term which for the present purposes will be considered to encompass those temperatures near the freezing point of water. Insects are separated into two broad groups depending on their ability to tolerate freezing. Those which tolerate freezing are said to be frost resistant. Insects which avoid freezing by supercooling are said to be cold resistant or cold-hardy (Wigglesworth, 1950). These two groups are synonomous with Salt's groupings, freezing resistant and freezing susceptible (Salt, 1961).

Originally, it was thought that increased polyol levels occurred

only during diapause in the Insecta. However, in both the adults and the eggs of the non-diapausing carpenter ant, Camponotus pennsylvanicus (L.) fluctuations of the glycerol levels are entirely temperature dependent, even during the summer season, and vary inversely with the ambient temperature (Dubach et al., 1959). Thus, in cold resistant insects, such as the carpenter ant, increased levels of polyhydric alcohols can be produced 'artificially' by chilling (Asahina, 1966). Therefore, diapause is not essential for polyhydric alcohol accumulation. Neither is the stress of cold temperatures necessary to begin glycerol accumulation, but rather this phenomenon of polyol accumulation is strictly dependent upon the species and the physiological condition of the developmental stage being considered.

Further evidence of this phenomenon, but contrary to the original belief that polyol accumulation was restricted solely to the Insecta, has been obtained by Somme (1965b). This report is of particular interest because the experimental species was not an insect but an arthropod related to the Insecta, the eggs of the European red mite, Panonychas ulmi (Koch.). It has been shown that the mite accumulates sorbitol during the fall. This finding is interesting because the Acarina, an order of the class Arachnida, exhibits a phenomenon which appears in the Insecta even though belonging to a different group of organisms. Therefore, this phenomenon may be more generalized than previously thought. Further physiological work with the Acarina may add numerous species which will accumulate polyols when cold-stressed. It was also believed that all frost resistant insects accumulate polyols. However, present information indicates that the possession of glycerol or

sorbitol by an insect is not a prerequisite for exhibiting cold or frost resistance. For example, Dubach et al. (1959) found that the larvae of the wood-boring insect, Parandra brunnea (Fab.) and those of Osmoderma eremicola (Knoch.) do not contain glycerol. Although glycerol may well play a major role in the winter hardiness of insects it is evidently not the only agent which enables insects to survive the effects of cold or freezing temperatures.

For many years it has been accepted that insects represent the most highly evolved multicellular animals that survive freezing (Hanec and Beck, 1960). Since there has been no documentation of survival of frozen adult insects, it was commonly accepted that freezing tolerance was confined strictly to the immature forms (Asahina, 1966). However, recently it has been shown that the adult carabid beetle, Pterostichus brevicornis, collected in Alaska, tolerates freezing during the winter months (Miller, 1969). It was found that the rate of cooling prior to freezing is a critical parameter with respect to whether the organism survives the freezing process. Earlier work had shown that this carabid beetle accumulates glycerol during the winter season in excess of 25% of the body weight (Baust, 1968). However, Miller believes in view of the relationship between glycerol and freezing resistance, additional information is needed before it can be said that glycerol is the factor which gives this carabid frost resistance. However, this result clearly shows that the limiting factor in frost resistance in insects is not necessarily the developmental stage of the insect (Miller, 1969). Miller indicated that Pterostichus brevicornis is only one of several frost hardy species

he found in hibernation. Further research in this particular area of cryobiology might reveal many such adult species.

It has been demonstrated that the possession of small molecular substances in the hemolymph favors survival during freezing (Asahina and Tanno, 1964; Salt, 1961). Such substances are found in higher plants and are thought to serve a cryo-protective function. One of these is the sugar, sucrose. It is now believed that there are other neutral substances analogous to those in plants which are involved in the winter hardening of insects. For example, the frost resistant prepupae, Trichiocampus populi OKan., contains no glycerol or sorbitol (Asahina and Tanno, 1964). However, the disaacharide, trehalose, is found in concentrations ranging from 5-9% of the fresh body weight. Because this level is similar to that of many non-overwintering insects, it is possible that the trehalose concentration does not necessarily convey a major cryo-protective action by itself, even though it may be indirectly involved.

According to Wyatt and Kalf (1956) trehalose is the principle sugar present in insects. Recent reviews indicate that insects feeding on honey dew and nectar exhibit high glucose levels as well as high trehalose levels. The high glucose levels may reflect the time of feeding or it may represent a delay in the conversion of the food into stored energy sources for the insect. However, the function of high glucose levels in the hemolymph is open to speculation.

Chino (1957,1958) has shown that almost all the glycogen initially present in the diapausing egg of Bombyx mori (L.) is rapidly broken down with the onset of diapause. As the glycogen disappears, glycerol

and sorbitol accumulate. It was also shown that the resynthesis of glycogen from glycerol occurred with the termination of diapause. Later reports provided evidence for the presence of the polyol dehydrogenases and phosphatases (Figure 1, reactions) for the formation of sorbitol and glycerol, which are in support of Chino's earlier conclusions (Chino, 1960; 1961; Horie, 1967). However, these studies did not prove the actual pathway. Secondly, both pathways for the formation of glycerol and sorbitol are present not only in diapausing eggs but also non-diapausing and developing eggs in which polyols do not accumulate (Harvey, 1962). Most insect physiologists assume that glycerol is formed directly via the glycolytic enzymes from glycogen, but this has not been proven.

Interestingly, glucose is the only monosaacharide found in Bombyx eggs throughout diapause, and no oligosaccharides are in evidence (Chino, 1957, 1958). It is curious that thirty years ago Kuwana (1937) made a significant finding that acid hydrolysis of Bombyx hemolymph caused release of a reducing sugar from a non-reducing substance that was not glycogen. Wyatt has shown this to be trehalose, present in both Bombyx larvae and pupae. A reexamination of the qualitative experiments with respect to the sugars present in the hemolymph might possibly contradict the original results. Present knowledge would indicate tat many insects have at least trace amounts of trehalose in all stages. It would appear the work was possibly limited by the sensitivity of the analytical techniques employed.

The hemolymph composition is variable with respect to qualitative and quantitative aspects unless one speaks of specific species. Generally

the nectar and honeyfeeding insects have high glucose and fructose levels. For example, the hemolymph of the honeybee, Apis mellifera L. is exceptional in the high content of free reducing sugars, glucose and fructose, present in approximately the same concentrations as trehalose (Duchateau and Florkin, 1959). Discussion of the absence or presence of specific sugars in the hemolymph is irrelevant unless one speaks of specific insects under specific conditions (diet, stage of development, temperature, etc.).

It is believed that glucose is converted to trehalose to facilitate diffusion of glucose across the gut wall (Treherne, 1957). The passive transport reported for the Insecta (Treherne, 1958 a-c) is in direct contrast to the active transport of sugars in mammalian systems. In some species it has been shown to serve as a mobile energy source for flight (Clegg and Evans, 1961). In the silkworm larva, trehalose has been suggested as a carbon source for chitin synthesis during molting (Duchateau-Bossam, et al., 1963). Asahina and Tanno, (1964) have suggested that it also serves a cryo-protective role in some insects. It has been demonstrated that trehalose levels vary in different developmental stages and in all probability this compound has been adapted to utilization for diverse functions within the Insecta (Murphy and Wyatt, 1965).

In light of the information that the respiratory enzymes, cytochromes b and c, show no enzymatic activity during diapause in the H. cercropia (L.) (Shappirio and Williams, 1953), Chino presents a possible mechanism which could account for the accumulation of glycerol and sorbitol. For example, as a result of the inactivation of the electron transport

system in diapausing insects the transfer of hydrogen to molecular oxygen would stop. The hydrogen of reduced NAD^+ or TPN^+ would reduce dihydroxy acetone phosphate resulting in the accumulation of glycerol. With the termination of diapause a reactivation of reduced DPN and TPN is postulated; oxidation and phosphorylation of sorbitol and glycerol would then take place with complete degradation of the polyhydric alcohol pool (Chino, 1958).

Chino's hypothesis becomes questionable in view of the data obtained by Wyatt and Meyer (1959). In diapausing S. cynthia (Drury) no glycerol could be detected. It was indicated in the preliminary assays (diapausing H. cecropia (L.)) that soluble DPN-linked dehydrogenases remain active. Shappirio and Williams (1953) state that cytochromes b and c virtually disappear during diapause. Thus the dehydrogenase reports are contradictory. However, if Shappirio and Williams are correct, Chino's hypothesis stands; if Wyatt and Meyer are correct, Chino's hypothesis becomes questionable. Since the accumulation of glycerol in H. cecropia (L.) tissues appears to be a characteristic feature of diapause as opposed to development, and in view of the above contradiction in formation, the reason for glycerol accumulation will warrant further analysis.

A second possible mechanism which may explain the accumulation of glycerol and sorbitol is that low temperatures inactivate specific enzymes. Jarabak et al. (1966) have demonstrated that human 17-hydroxysteroid dehydrogenase loses activity rapidly below 10-15°. The addition of 20% or more of glycerol protects the enzyme against cold

inactivation. There may be an analogous enzyme in the glycolytic pathway which is initially cold inactivated, resulting in a shift in the steady state and the accumulation of glycerol. However, as the glycerol accumulates it may act as a feedback system, partially activating the enzyme, and thus resulting in maintenance of a constant level at a specific temperature.

Since the initial reports of Chino (1957) and Wyatt and Kalf (1958), many different approaches have been attemped to ascertain the function of polyols in cold and frost resistant insect species. It is evident that one of the functions of polyhydric alcohols in insects is to increase the ability of cold resistant species to supercool, that is, to lower the temperature at which ice crystals form in the hemolymph, below that of the normal freezing point. Such processes relating the effects of glycerol on the supercooling points of insects have been studied in several species (Salt, 1959; Somme, 1964, 1965a). It has been demonstrated with some insects that the glycerol concentration of the hemolymph is inversely proportional to the supercooling point. There is no real evidence that this phenomenon is not detrimental to the insect.

Another approach to studying the function of polyols in insects is to investigate the influence of various injected substances on the hemolymph composition and concentrations of various solutes. Somme (1966, 1968b) demonstrated that oral injection of glucose and trehalose, (5 ul. of a 40% Ringers solution), lowered the supercooling point. Earlier, Tanno (1964) had suggested a relationship between low supercooling points and high levels of glucose, fructose, and trehalose in overwin-

tering solitary bees. Thus, Somme's (1968b) results support Tanno's (1964); hemolymph sugars also have capabilities of lowering the sugar cooling points. Calculations show that at physiological concentrations, these substances could not account for more than a maximum total drop in the freezing point of 3°.

Since some insects can survive freezing without glycerol or sorbitol, experiments have been conducted to determine the direct effect of glycerol on frost resistance and cold resistance by injecting glycerol into freezing-susceptible insects. When glycerol was injected into a cold resistant species, they were not made frost resistant (Asahina, 1966). One would expect that an increased glycerol level might lower the supercooling point, but would probably not convey frost resistance.

The effects of acclimation on species with normal glycolytic levels of polyhydric alcohols is another area of active investigation. As indicated by several authors (Prosser, 1967; Somme 1966, 1968b), acclimation of insects at various low temperatures results in two major responses. Some show increased activity and metabolic rate when acclimated at low temperatures within their normal temperature range, while others display an increased ability to tolerate more extreme temperatures as compared to unconditioned specimens.

The effects of acclimation on insect survival were first noted by Mellanby (1939). Colhoun (1960) found high mortality in specimens of Blattella germanica (L.), the German cockroach, which were moved directly from 35° to 7°, as compared to those which were first conditioned at 15° or 25°, and then moved to 7°. The results show that for the acclimation temperature range of 10° to 35°, the chill-coma temperature,

(the temperature at which the organism is incapable of standing up or crawling), for the species is lowered by 27°. Colhoun assumes that conditioning prior to cold exposure was the factor that enabled the specimens to survive longer. It is questionable whether such slight modification in response to cold stress would be of value to Blattella as a survival factor. Secondly, Blattella has a gradual metamorphosis; in the experiments specimens of various ages were used and this might introduce error. As found in the holometabola, different developmental stages show different responses to cold stress, thus age effects could also be true in the paurometabola.

Using larvae of Ephestia kuehniella (Zell.), Somme (1966, 1968b) demonstrated that acclimation lowers its supercooling point. Concurrent with this increase in supercooling, hemolymph concentrations of several solutes were elevated (Somme, 1966). While glycerol and glucose levels increased at 0°, the concentration of trehalose increased at 0° but decreased at -6°. Further experimentation with Ephestia kuehniella (Zell.) demonstrated that oral injection of glycerol also resulted in a depression of the supercooling point (Somme, 1968b). Oral injection of glucose and trehalose further demonstrated that substances other than glycerol may effect supercooling. Somme (1968b) demonstrated that temperature is a limiting factor in the acclimation process. If the organism is stressed with a severe temperature, there is no lowering of the supercooling point as compared to unconditioned specimens stressed with cold. Instead, specimens react to supercooling temperatures as if they were not conditioned. This reflects the physiological limits to which an insect may adjust.

It is interesting to note Somme's (1968) data on pupation of acclimated larvae. Even though larvae orally injected with glycerol survive lower temperatures in greater numbers than controls, (expressed as percentage of the numbers surviving the temperature treatment), fewer that are orally injected survive to pupate. Secondly, of those that survived the stress of sub-zero temperatures, but which had not been cold acclimated exhibit the highest percentage of pupation. This data would suggest that even though the glycerol treated specimens could withstand lower temperatures than the others, this was a pseudo-physiological condition which did not allow the organism to develope normally.

Evidence shows there is a relative temperature below which the overwintering stage will succumb if exposed for very long periods. For example, colonies of Apis mellifera L. must maintain a minimal temperature of 57°F or death will result (Eckert and Shaw, 1960). The carpenter ant, Camponotus pennsylvanicus (L.) can tolerate temperatures below 0° in a supercooled state for periods up to 5 months as long as there is no internal ice formation (Dubach, et al., 1959). Another example is the larvae of the European corn borer, Ostrinia nubilalis (Hübner): it can be frozen and thawed without any visible detrimental effects (Hanec and Beck, 1960). Injury is likely to result if the body temperature of an insect drops below the minimal level for any prolonged period of time. However, Salt (1966) has demonstrated there is a maximum length of time an insect can tolerate a specific temperature. This time is directly proportional to the temperature of the environment: the lower the temperature the shorter the period before

detrimental effects can be observed in the organism. Thus, the minimal temperature which an insect can tolerate without injury depends on the species considered and the stage of development. Other factors affecting the minimal temperature which an insect can survive is the length of time of exposure, pre-conditioning of the specimen, and the rate at which the temperature is decreased.

MATERIALS AND METHODS

The following chemicals were purchased from Sigma Chemical Company: nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP), α-glycerol phosphate (α-GP), dihydroxyacetone phosphate (DHAP) (as the dimethylketal dimonocyclohexylamine salt), and iodoacetic acid as the free acid. Uniformly labeled C-14 Glucose, specific activity 190-210 mc/mM, was obtained from International Chemical and Nuclear Corp. Glycerol and Triacetin (Glycerol triacetate) were bought from Eastman Chemical. Scintillation grade POPOP, and PPO were purchased from New England Nuclear Corp. Chromosorb Q (120 mesh) support and ECNNS-M liquid phase were obtained through Applied Science Laboratory Inc., State College, Pa. Other chemicals used were reagent grade.

Amberlite IR-120 (H^+) and IR-45 (OH^-) were obtained from Mallinkrodt. Whatman No. 1 chromatography paper was purchased from Reeve Angle Co. Inc. Pre-made silica gel thin layer chromatography plates (TLC-Plates), manufactured by E. Merck A.G. Darmstedt Germany, were ordered from Brinkman.

The following solvent systems were used during the course of this study. Solvent A: pyridine, ethyl acetate, water-2:5:7 v/v upper phase; B: water saturated phenol; C: isopropyl alcohol, ascetic acid, water-54:8:18 v/v; D: hexane, ethyl ether, ascetic acid-100:10:1 v/v; E: n-butanol, pyridine, water-6:4:3 v/v; F: pyridine, ethyl acetate, water-7:1:2 v/v.

Paper chromatograms were developed in the following way. The dried chromatogram was dipped in silver nitrate solution which contained 10ml. of saturated silver nitrate in 1 liter of acetone. After drying it was dipped in a solution which was a 0.75M solution in KOH. The moist alcohol paper was finally dipped in aqueous thiosulfate.

Paper chromatographs were also developed by spraying with aniline hydrogen phthalate solution when aldoses were being differentiated from ketoses. The solution was made by dissolving 0.93g anailine and 1.66g phthalic acid in water-saturated n-butyl alcohol. Spots were detected under U.V. light: aldophentoses showed red, aldohexoses brown, and ketoses do not show up (Partridge, 1949). The scintillation fluid consisted of 10g of 2,5-diphenyloxazole benzene dissolved in 1.6 liters of toluene. To this was added one liter of Absolute alcohol.

Insect Cultures. The hibernating Ichneumons were collected from both western Massachusetts and Ohio in the fall and winter months. Specimens were found hibernating in rotten logs and stumps, under bark, between rocks, and under moss carpents. Non-hibernating forms were collected in Massachusetts during the spring and summer. Upon collection, specimens of either non-hibernating or hibernating forms were brought back to the laboratory for immediate experimentation.

All ants (Camponotus pennsylvanicus (L.)) used in these experiments were collected from wooded areas in western Massachusetts. Two cultures were usually maintained, one in the cold at 4^{o}, and one at room temperature. When ants were brought in from the field, they were placed in the stock culture in natural galleried logs in an open container at 4^{o}. It was

in this culture that the reserve supplies of ants were kept. The working culture, which was kept at room temperature in a screen-covered aquarium filled with galleried wood, was the stock from which ants were taken in order to perform experiments.

To replenish the working culture, ants were removed from the stock culture. These ants were allowed to acclimate themselves metabolically a few days before being used in experiments. Early experiments showed glycerol levels fall to normal from the high cold stressed levels in a matter of a few days.

The working cultures had to be supplied with both food and water while the stock culture required none. A wet sponge supplied a continual source of water. Ants were periodically given honey as a source of food and protein.

It was found that ants could be maintained in the stock culture for up to six months without an observable detrimental effects. Cold storage always resulted in some mortality, and prolonged storage greatly increased this. Storage for over six months in the cold resulted in observable cryobiologically induced changes, including mortality and lack of coordination.

Extraction Procedure. Prior to extraction of metabolites, all experimental specimens were weighed to the nearest tenth of a milligram. After weighing, the ants were placed in a glass homogenizer with approximately 4-5ml. of methanol:water (3:1 v/v) solution. (Water was used when glycogen was extracted). The mixture was ground until the exoskeleton was broken into fine pieces. The solution was then centrifuged at 4,000 rpm for 5 minutes (Sorvall RC2-B Refrigerated

Centrifuge). The supernatant was poured off and the pellets in the bottom resuspended with the water-methanol solution. Again the mixture was spun down at 4,000 rpm for 5 minutes. The supernatants were combined and the pellet discarded.

The solution was then washed three times with 2ml. of ethyl ether to extract the lipids. After allowing the ether-extract to stand a few minutes, the ether was drawn off and kept in a separate container. Sometimes an emulsion formed between the ether water layers. This could be resolved by spinning the emulsion at 4,000 rpm. Ether and water phases formed with a thick interface between them. This may arise from an insoluble wax extracted from the cuticle. The ether solution was then back-extracted twice with water. This water solution was then added to the original supernatants.

Following the back extraction of the ether-lipid fraction, the aqueous extract was cooled to 0°, and the protein was extracted with 5ml. of 10% TCA (final concentration 5%). The protein precipitate was centrifuged at 5,000 rpm for ten minutes. The clear supernate was drawn off and extracted with 2ml. portions of ethyl ether until the TCA was removed, as evidenced by monitoring the pH.

The solution was then cooled again to 0°, and to this was added 1½ volumes of ethyl alcohol. The solution was allowed to stand so that the precipitated glycogen could flocculate. The suspension was spun at 5,000 rpm for ten minutes at 0°. The supernatant was removed and the glycogen kept in an alcohol suspension until needed.

Identification and Quantitation of Glycerol. In experiments where glycerol was the only carbohydrate being analyzed, the solution containing

the insect extracts were filtered through glass wool and evaporated to near dryness. The solutions were deionized on a mixed bed ion exchange column (0.5 x 2 in.) of Amberlite IR-120 (H^+) and IR-45 (OH^-). Each effluent was taken to dryness under reduced pressure in a 12ml. conical centrifuge tube and acetylated with 0.25ml. pyridine and 0.25ml. acetic anhydride at 70° for 20 minutes. Excess pyridine and acetic anhydride were removed under vacuum and suitable aliquots of acetylated extracts were analyzed by gas chromatography (Perkin-Elmer Model F-11 Gas Chromatograph), which was carried out using a 10 ft. x 1/8 inch stainless steel column with Chromosorb Q support and a 3% ECNNS-M liquid phase, run isothermally at 135°. Retention times, detected by flame ionization, were determined with a strip chart recorder (Honeywell, Model 19 Recorder). Over the range of temperatures examined, the only compound to elute from the column in significant quantities was glycerol triacetate. Its identity was verified by comparison with standard glycerol triacetate. The retention times of the two were identical. Trace components were detected in all samples at high recorder sensitivity but these accounted for only one or two percent of the material eluted. Since sorbitol has been detected in overwintering insect species, attempts were made to identify the hexacetate of the polyol. However, none could be detected in any one of the extracts.

Quantitation of the results was accomplished using known concentrations of standard glycerol triacetate solutions. The areas under these peaks were determined and found to be directly proportional to the quantity of standard injected. All determinations were made in

the one to seven microgram range.

Experiments designed to test the reproducibility and the efficiency of the extraction and acetylation process were formed by adding known amounts of glycerol to extracts of warm insects which contained no appreciable quantity of glycerol. Acetylation of the extracts and removal of excess pyridine and acetic anhydride showed a recovery of 85% of the glycerol originally added to the samples.

The glycerol fraction in experiments involving the separation of glycerol from the sugar components was treated differently from above. The clear extract was deionized on a mixed bed ion exchange column (0.5 x 2 inches) of Amberlite IR-120 (H^+) and Amberlite IR-45 (OH^-). After evaporating the solution to near dryness, the extract was streaked on Whatman No. 1 Chromatography paper. Descending paper chromatography was run for approximately 24 hours in solvent A. The glycerol area was identified by developing the standards, marking out the glycerol area, and cutting it into pieces, and soaking in 5ml. ethanol:water (1:1 v/v) in a 50ml. flask. This was repeated three times. The supernatant was collected and evaporated to dryness. It was then quantitated as previously outlined.

The dry strips of paper containing the glycerol standards were developed using the silver nitrate method (Trevelyn, et al., 1950).

The identification of glycerol isolated from cold stressed ants was also confirmed by paper chromatographic analysis of deacetylated extracts. To the glycerol triacetate, 1ml. of methanol and a small chip of metallic sodium were added. The solution was deionized, evaporated to dryness, and spotted on a paper chromatogram (Whatman No. 1)

run in solvent A. A spot was detected which had the same mobility as that of standard glycerol.

Identification and Quantitation of Glycogen. Ants were analyzed for their glycogen content, following the precipitation of protein; the glycogen was precipitated with 1.2 volumes of ice cold ethanol. The polysaccharide was allowed to flocculate and precipitated in a 12ml. conical centrifuge tube in crushed ice. Each sample was subsequently reprecipitated twice from two ml. of water by slow addition of four ml. of ice cold absolute ethanol. The glycogen was finally dissolved in 5ml. of water and its concentration determined in a suitable aliquot by phenol sulfuric acid method (Dubois, et al., 1956). The tubes were read after 15 minutes on a Coleman Junior spectrophotometer at 490 mu. Readings of known concentrations of glucose were used to calculate the concentrations of glycogen solutions.

Sugar Composition of the Hemolymph. Extraction was carried out as previously described. The water extract was concentrated and spotted on Whatman No. 1 chromatography paper and run in solvents A and B. Solvent C was used to differentiate glucosamine and trehalose.

Standard solutions (5%) of glucose, fructose, maltose, arabinose, glucosamine, n-acetylglucosamine, erthrose and trehalose were also run.

Visualization of spots was accomplished using the technique of Trevelyn et al., (1950), and also a technique specific for the differentiation of ketoses from aldoses (Patridge, 1949).

Purification of ^{14}C-Glucose. Uniformly labeled ^{14}C-glucose was purified by chromatography of the sugar on Whatman No. 1 in solvent A. The radioactive material was eluted from the chromatogram with water

and evaporated to dryness. The material was diluted such that there were approximately 100,000 cpm per ul. To prevent self decomposition and possible bacterial degradation, it was then frozen until it was used.

Radioactive Incorporation. For the experiments involving radioactive incorporation female reproductives were used because of their larger size. Two microliters of uniformly labeled ^{14}C-glucose in water, (approximately 100,000 cpm/ul), were injected internally into the abdomens of the female reproductives. Two people were required to perform the injections. One person would hold the ant while the second injected the ant in the membranous region between tergites 5 and 6 with a 10 ul. Hamilton syringe. The tip of the syringe was inserted approximately a quarter of an inch and then withdrawn slowly after the 2 ul. were released. There appears to be little fluid loss at the point of entry.

Extractions for various substances were carried out as previously outlined. However, the lipid fraction (ether solution) was further separated so that the triglycerides could be isolated. The triglycerides were isolated by streaking the lipid fraction on silica gel TLC plates along with standards and run in solvent D. The triglycerides were detected by spraying the standards with a 10% Rhodamine solution and the use of an ultra-violet light. The triglycerides were detected as pink spots under the U.V. light. The unknown triglyceride area was scraped from the plate, eluted twice with 2ml. of ethyl ether, and evaporated to dryness.

Glycerol, the triglycerides and the sugars could be counted directly. Glycogen had to be hydrolized before it was soluble in the

counting fluid. To two ml. of the glycogen solution, 0.4ml. of 10 N H_2SO_4 was added and heated in the boiling water bath for fifteen minutes. This solution was neutralized with $BaCO_3$. After centrifuging the milky $BaCO_3$ solution for five minutes at 10,000 rpm, the clear supernatant was drawn off and used for quantitation purposes.

Counting was done using 10ml. disposable glass counting vials with a Packard (Model 3310) liquid scintillation spectrophotometer. To 5ml. of scintillation fluid was added 10 ul. of unknown. The unknown was counted for at least 10,000 counts or 100 minutes (maximum time per vial). A blank was always run with any set of vials being counted. The blank was used as a background count and subtracted from all data obtained.

Assay of Enzyme Activities. The assay for ∝-glycerophosophate phosphatase was performed by measuring the amount of inorganic phosphate released by thehydrolysis of ∝-glycerophosphate. Phosphate was quantitated by a modified Fiske-SubbaRow method. The presence of alcohol dehydrogenase and ∝-glycerophosphate dehydrogenase were determined by observing the rate of reduction of nicotinamide adenine dinucleotide (NAD^+) in the presence of the respective substrates. Absorbance changes were measured at room temperature in a Gilford 240 spectrophotometer, and monitored with a Honeywell (Model 19) strip chart recorder.

∝-Glycerophosphate Phosphatase.

$$\text{∝-GP} \longrightarrow \text{Glycerol} + \text{Pi} \quad (1)$$

∝-glycerophosphate phosphatase was assayed according to a modified

method of Leloir and Cardini (1957). The ants were weighed and placed in a chilled hand homogenizer with 1ml. of Tris-acetate buffer (0.01M, pH 6.5). The mixture was ground until the exoskeleton was pulverized. The solution was then centrifuged at 4,000 rpm for 5 minutes (Sorvall RC2-B Refrigerated Centrifuge). The supernatant was drawn off and the pellet discarded. This was repeated again and the supernatants combined. From this solution a control was prepared with 0.2ml. of extract, and 0.2ml. of 10% TCA. This was spun at 15,000 rpm for 20 minutes. The reaction mixture was prepared with 0.4ml. of extract, and 0.3 ml. of α-glycerophosphate (15mg./ml.), and incubated at 30° for varying lengths of time. Samples (0.35ml.) were drawn off at specific time intervals to which was added 0.35ml. of 10% TCA. This was centrifuged at 15,000 rpm for 20 minutes.

Inorganic phosphate was determined according to a modified Fiske SubbaRow method. To each sample the following were added: 0.2ml. 1ON H_2SO_4, 0.2ml. of 5% ammonium molybdate, and 0.05ml. Fiske SubbaRow reagent. This was then heated in a boiling water bath for 7 minutes. The samples were then read at 660mu in 0.05ml. cells in the Gilford spectrophotometer. Readings of known concentrations of inorganic phosphate were used to calculate the amount of phosphate present. Subtracting the amount of phosphate present in the control from the reaction mixture gave an index of the amount of enzymatic activity.

Alcohol Dehydrogenase.

$$CH_3CH_2OH + NAD^+ \longrightarrow CH_3CHO + NADH + H^+ \quad (2)$$

Alcohol dehydrogenase was assayed according to the method of Kersters and DeLey (1966). Ants were weighed and placed in a chilled

hand homogenizer with 2ml. phosphate buffer (o.1 M, pH-7.0 and 0.01M mercaptoethanol) and ground until the exoskeleton was broken into small pieces. The extract was centrifuged at 5,000 rpm for 20 minutes (Sorvall 11 RC2-B), the supernatant was poured off and the pellet discarded. This was repeated again and the two supernatants combined and placed on ice. After dialysis against phosphate buffer (0.1M, pH 7) the extract was assayed. To the reaction mixture was added: 0.45ml. buffer, 0.05ml. $MgCl_2$ (0.05M), 2ul. NAD^+ (0.5u mole/ml.), 5ul ethyl alcohol, and 100ul enzyme extract. Absorbance changes were then measured with the spectrophotometer at 340.

<u>∝-Glycerophosphate Dehydrogenase</u>.

$$DHAP + NADH + H^+ \longrightarrow \alpha\text{-}GP + NAD^+$$

∝-glycerophosphate dehydrogenase was extracted according to the following procedure. Ants were weighed and placed in a chilled hand homogenizer with 2ml. tris buffer (0.1M. pH 7.5, 2g/L. EDTA) and ground until the exoskeleton was broken into fine pieces. It was then centrifuged at 5,000 rpm for 20 minutes, and then at 15,000 rpm for 20 minutes (Soverall 11 RC2-B). The supernatants were poured off and the pellet discarded. This process was repeated and the supernatants were combined and placed on crushed ice. The assay mixture contained the following: 1ml. glycine buffer (0.1M pH 10.2), 0.1ml. extract, 6 ul NAD^+ and 6 ul ∝-GP. The reaction was also measured in the reverse direction going from DHAP to ∝-GP. The reaction mixture contained: 0.9ml. Tris buffer pH 7.5, 0.1ml. Iodoacetic acid (0.1M), 2ul NADH, 5ul DHAP, and 0.1ml. extract. The reaction was followed by measuring absorbancy changes at 340 mu.

RESULTS AND DISCUSSION

The reviews of Salt (1961) and Asahina (1966) document numerous investigations which have studied the mechanisms of winter survival and polyol accumulation. Various immatures and adults of species from several orders of insects have been used in making the comparison of results feasible only at a low level of specificity. Thus, until now, no one has executed experiments designed to ascertain whether the overwintering ability of a group of closely related species is manifested by a common observable phenomenon, namely polyol accumulation, and whether this phenomenon is restricted to those species which hibernate, while closely related, but non-hibernating species do not exhibit this behavior. For the present investigation, species from the Ichneumoninae (Ichneumonidae, Hymenoptera) were used because 12 genera out of over 250 are most unique in that they are the only members of the entire family, with the exception of the genus Orthocentrus (Townes) which are able to pass the winter as free living adults. Curiously, only the fertilized females of these genera are found hibernating during the winter; males do not overwinter (Heinrich, 1960). One would expect a species capable of overwintering as adults to possess some unique physiological mechanism which is lacking in non-hibernating species in the family.

Experiments were run where all specimens were cold stressed at 4° for three weeks, following a period at room temperature in the laboratory. Results are shown in Table 1. Only hibernating species are capable of low temperature glycerol accumulation with the exception

of two males of hibernating species. This work represents an attempt to examine closely related species with the purpose of ascertaining whether there is an observable relationship between adult hibernating and non-hibernating species and glycerol accumulation. The results clearly indicate that the ability of Ichneumons to hibernate as adults is coupled with their ability to accumulate glycerol. Reports of two Ichneumoninae species from the Eastern Paleartic region which produce glycerol when stressed with cold, Hoplismenus obscurus Kriech., and Ichneumon molitorius L., further support this view (Takehara, 1966).

Assuming there is survival value in the ability of some insects to accumulate glycerol, one must attempt to assign some role to the phenomonen in the life-cycle of this insect. Interestingly, the reproductive behavior of these insects offers one possible use for glycerol. After the hibernating species mate in the fall the male dies and the female stores the spermatozoa in the abdominal spermatheca for a period of 4-5 months until the spring, when it leaves its hibernaculum in search of pupae to parasitize (Heinrich, 1960). It is believed that after the female leaves her hibernation location, the spermatozoa are released from the spermatheca to fertilize the egg prior to oviposition. This may be important in view of the fact that glycerol is a very good cryo-protective agent for long term storage of bovine spermatozoa at sub-zero temperatures (Polge et al., 1949). To assess this possible role of glycerol as a cryo-protective agent in hibernating insects, experiments must be performed to determine the composition of the fluid of the spermatheca. Secondly, experiments must be designed to test whether the presence of glycerol or other cryo-protective agents,

which may be present, have any affect on the fertilizability of the spermatozoa.

It was evident that after working with the Ichneumons further experiments would demand a larger number of specimens than was feasible. Thus, the carpenter ant, Camponotus pennsylvanicus (L.) was chosen because of the availability of large numbers of the ants at a minimal cost.

Throughout the course of this study, working with the ants resulted in a number of random obversations. It was found that in replenishing the working culture, higher mortality was obtained if the galleried logs were disturbed such that the hibernating ants fell out. By allowing the ants to cluster in the natural galleried wood, considerably less mortality was observed. This may possibly indicate that the clustering phenomenon of the ants is a beneficial behavioral pattern which aids in survival during hibernation. It was also evident that male reproductives were the least hardy of the ant castes, and that the female reproductives were the hardiest. Activity in room temperature colonies was most noticeable at night. Seldom could female reproductives be seen feeding, but rather would be fed by the workers. Occasionally, female reproductives could be seen trying to fly. Another interesting observation was that ants taken from one colony in the field could not be mixed with ants from another colony; fighting would result. By chance it was found that the ants are very sensitive to butyric acid. The fumes from butyric acid being used in the laboratory caused the ants in the working culture to issue forth from the galleried wood. Within one hour the entire colony was dead.

Collecting in the field is simplest during the winter months because the ants are immobile. When collecting during hibernation, ants could be seen in galleries partially filled with ice crystals. This raised the question of whether Camponotus pennsylvanicus (L.) can tolerate freezing under optional conditions, while literature states they cannot (Dubach et al., 1959).

The initial collection and storage temperatures may be crucial to a meaningful study. If insects are stored at "warm" temperatures, even though below their freezing points, a frost resistant state is tenuous. Glycerol may degrade rapidly, but may still appear to the investigator to be at "high" levels. Therefore, these levels may be below those required for functional frost resistance. This would result in the erroneous conclusion that a given species is not frost resistant. Preconditioning during the fall may be another important factor in enhancing the ability of the ants to survive low temperatures.

Initial experiments with Camponotus were conducted to examine the more generalized aspects of glycerol synthesis and accumulation. In order to ascertain whether glycerol was localized in a particular body region of female reproductives (Camponotus pennsylvanicus (L.)), the glycerol contents of three body regions (head, thorax, and abdomen), were analyzed separately by gas chromatography. For this experiment female reproductives were frozen in dry ice powder immediately before dissecting them to eliminate loss of hemolymph. Table 2 indicates that all body regions contain about the same amount of glycerol. A disproportionate distribution is not suggested. It was considered that because the abdomen contains the ovaries and the site of egg

storage, perhaps there might be a higher level present there than in the head or thorax. It has been suggested with a similar phenomenon in hibernating Ichneumoninae, (Ichneumonidae, Hymenoptera), that the spermatheca in the abdomen may have high glycerol levels with the possible role as cryoprotective agent for the semen in this hibernating group (Duffield and Nordin, 1970). However, high glycerol levels were not detected in the abdomen. Secondly, this datum does not suggest a generalized site of glycerol synthesis. It is possible that there is a specific site for glycerol synthesis which releases the polyol into the circulating hemolymph.

Table 3 illustrates glycerol levels in each caste of Camponotus. Female reproductives, soldiers, and workers all produce glycerol at -1°. Interestingly, all of the male reproductives died during this experiment. However, significant qualities of glycerol in the male reproductives have been detected at 4°. From experience in storing male reproductives in the laboratory, they are the least resistant to the cold of the castes. Wheeler (1911) states that they are not usually found in overwintering colonies. However, in collecting Camponotus in the field, it was found that a well established overwintering colony will have both male and female reproductives. Old colonies will have all male reproductives, indicating that the colony has an old queen. In young colonies, (less than 1,000 individuals), there will be a queen and possibly one or two dozen reproductives (male and female), and the remainder will be made up of workers and soldiers.

Changes in glycerol levels as a function of cold stress were measured

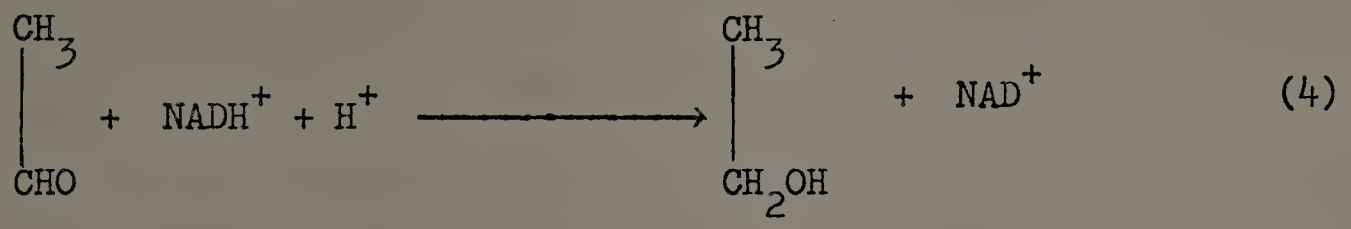
since work on other insects suggests that glycerol arises from the breakdown of glycogen (Chino, 1957; Takehara, 1966). Results shown in Table 3 also indicate that the glycogen levels in Camponotus pennsylvanicus (L.) decrease during hibernation. However, this only suggests that glycerol may arise from glycogen at low temperatures in this organism. Previous investigations (Asahina, 1966; Dubach et al., 1959) have shown that glycerol buildup varies inversely with temperature, as measured during monthly or seasonable periods. No data, however, is available on the initial rates of accumulation. Since these initial rates could tell something of the process involved, glycerol buildup following cold stress was examined over two time periods. Figure 2 shows an approximately linear increase in glycerol concentration in the ants maintained at -1°, examined on a weekly basis. Since the data suggest a possible lag early in the stress period, it was obvious that data was needed for earlier time periods. By analyzing ants every 12 hours during exposure, at -1°, results were obtained that show there was a significant accumulation within 12 hours (Figure 3). It has been pointed out (Nordin et al., 1970) that "this mechanism of accumulation is quite remarkable when one considers that in nature the organism does not have to respond as rapidly to changes in environmental temperature as they do in the experimental period of cold stress." The results clearly indicate a rather linear glycerol accumulation within the limitations of the analytical procedure. However, this data does not rule out the possibility of a short lag period.

Figure 4 indicates that -4° is the lower limit at which ants

produce glycerol under the conditions of the particular experiment. It would appear that glycerol synthesis is inhibited at -3° and below. Mortality began to increase drastically below these temperatures. At -6° all the ants died shortly after beginning the test period. The decreased glycerol levels present at -3° and below would imply that its accumulation is being offset by inactivation of other body processes. These results indicate that -6° is the lower temperature limit at which Camponotus pennsylvanicus (L.) can survive under specified conditions without preconditioning. However, it is clear that the ants do not make more glycerol at a lower temperature than at a higher temperature for the same time period (within -3° to +4° range).

The question has arisen whether fluctuations in the glycerol levels might not be a result of cold activation or inactivation of specific enzymes which commence functioning at a low ambient temperature (Nordin et al., 1970; Baust and Miller, 1970). In the light of the information on the inactivation of specific enzymes in the cold (Jarabak et al., 1966 Stancel and Deal, 1969) experiments were run which involved assaying for differences in enzymatic activity between crude extracts of warm and cold ants. Assays were performed for alcohol dehydrogenase, ∝-glycerophosphate dehydrogenase, and ∝-glycerophosphate phosphatase. Alcohol dehydrogenase was assayed to see if an anerobic mechanism similar to that devised by Neuberg for the commercial production of glycerol was operative. In his system a bacterial fermentation takes place in the presence of bisulfite. The bisulfite combines with acetaldehyde which results in the block of

alcohol dehydrogenase. The lack of an acceptor for NADH shifts glucose metabolism so that glycerol accumulates via the utilization of ∝-glycerophosphate dehydrogenase (Figure 1). For glycerol to accumulate under anerobic conditions, the following reaction, catalyzed by alcohol dehydrogenase (Dixon and Webb, 1965), would have to be blocked.



(4)

Thus the presence of alcohol dehydrogenase would provide a possible reaction that could be cold inactivated and allow for glycerol buildup. However no alcohol dehydrogenase activity could be detected in Camponotus. In attempts to find activity, the coenzyme $NADP^+$ was substituted for NAD^+. This had no effect. Considering the insect system, alcohol dehydrogenase might require a different metallic ion, thus Mg^{++} was substituted for Zn^{++}. This also gave negative results. The results were not altogether surprising when one considers that the literature reports only measurable levels of this enzyme in yeast (White et al., 1968).

∝-glycerophosphate dehydrogenase and ∝-glycerophosphate phosphatase were considered because, as seen in Figure 1, if either of these enzymes were activated in the cold, there would be a shift in the steady state from G-3-P toward DHAP, resulting in an increased glycerol level. Crude enzyme extracts prepared from both cold and warm ants showed no difference in ∝-glycerophosphate dehydrogenase activity, thus indicating that the enzyme is not more active in the cold than at room temperature. Because of the problems with extraneous inorganic phosphate present, the

∝-glycerophosphate phosphatase assay did not give meaningful results. The possible breakdown of the sugar phosphates, and other phosphate compounds in the crude extract, made it impossible to tell if one was measuring ∝-glycerophosphate degredation. A more purified enzyme preparation will be needed for future studies.

Experiments involving incorporation techniques using labeled compounds are one means for ascertaining the sources of glycerol. The injection of specimens with unlabeled glucose solutions indicated that seldom did an ant succumb shortly after injection. Injury did not appear to be a problem. Fluid was occasionally observed leaking from the point of entry. This leakage does limit the technique because it was assumed that all experimental specimens initially received the same number of counts when isotope was administered.

Chino (1957, 1958) has reported that nearly all the glycogen initially present in the diapausing eggs of Bombyx mori (L.) was rapidly converted to sorbitol and glycerol. This report is based on the fact that glycogen levels fall as glycerol levels rise, and with the termination of diapause glycogen is resynthesized from these polyols. However, no real experimental proof is given in Chino's reports. Because each step in sugar metabolism has alternative paths, it is possible that glycerol may initially arise from a sugar such as glucose or trehalose, or possibly from lipid, for example the triglycerides. Therefore experiments involving labeled precursors were used to try and trace the path of glycerol synthesis. By comparing the total activities of isolated glycogen from ants both at room temperature and in the cold, differences found in the total activities should be

accounted for by the activity found in the glycerol fraction supporting Chino's results. Ants were injected with ^{14}C-glucose (approximately 200,000 CPM) and placed immediately at -1° to resolve which compounds were being labeled, and what effects various lengths of time in the cold had on the specific activities of various compounds. Table 5 indicates that as glycerol accumulates during the first 8 hours it becomes heavily labeled. As time progresses, the CPM/mg ant begins to fall. A possible explanation for the initial labeling pattern may be the result of ^{14}C-glucose moving along the Embden-Meyerhof pathway via the glycolytic enzymes to glycerol. Figure 1 indicates that glucose may also go to other products. However, as the ant begins a cryotaxic response, there is a change in the glycolytic pathways evident by the accumulation of glycerol. The ant begins its physiological and biochemical responses to the cold stress; the breakdown of unlabeled stored energy sources to form glycerol commences. These precursors may be glucose, trehalose, glycogen, or the glycerol may originate from the breakdown of the triglycerides. Thus, as glycerol levels increase, the total number of counts remains constant, and the CPM/mg ant should remain constant. This is not the case with the counts, indicating a turnover of glycerol may be taking place.

The glycerol from the saponification of the triglyceride fraction showed very little incorporation (Table 6). Because the counts were extremely low, it appears that the carbon skeleton of glucose is not being converted to triglyceride glycerol <u>before</u> appearing as free glycerol, but is most likely being converted directly to glycerol, via glycolysis.

Glycogen, like glycerol, becomes labeled initially only to lose activity with time (Table 7). To present a feasible explanation, the structure of glycogen must be reviewed. Glycogen is a branched-chain polysaacharide, having 8 to 12 glucose residues per non-reducing end group (White, et al., 1968). The chains are made up of 1,4 glucosidic and 1,6 glucosidic bonds. With the initial injection of ^{14}C-glucose, some of the labeled glucose residues are added to the ends of the branched structure of glycogen by the actions of glucose synthetase and amylo-(1,4-1,6) transglucosoylase. Thus, only the more terminal residues of the glycogen molecule are labeled. As seen in Table 6 this process would appear to stop within 8 hours at -1^{o} as the ant begins a cryotaxic response to the cold stress, glycogen becomes an energy source. The enzyme, glycogen phosphorylase, degrades glycogen to -glycose-1-phosphate (Figure 1). As the terminal glucose residues are removed, the specific activity of the glycogen falls rapidly.

In light of the information presented in Tables 4,5,6 it becomes evident the triglyceride fraction is not heavily labeled. It also appears that glycogen and glycerol account for approximately half of the counts initially present. One questions whether glycerol does not arise from a carbohydrate source other than glycogen. Because the water soluable fraction contains approximately 09% of the initial counts, a divergent line of experimentation was undertaken. With this in mind chromotography work divulged that ants had high glucose and trehalose levels. Traces of maltose and fructose could

also be detected. Table 8 indicates the relative concentrations of glucose and trehalose.

With this basic information and the information from the first experiments, (Tables 2,3,4,5), the next experiments were designed differently. Because the labeled compound in the first experiment did not reach steady state conditions, it was felt the total counts in the various fractions might not be representative. Thus in the second experiment female reproductives were injected with 2 ul of ^{14}C-glucose, (approximately 200,000 CPM), and allowed to equilibrate at room temperature for 48 hours. Then three groups of 6 were placed at -1°, and two groups left at room temperature. The room temperature groups were employed as controls to indicate the "normal" distribution of the labeled compound with time. Ants were removed from the cold after 12,24, and 48 hours and sacrificed. Controls were sacrificed at 24 and 48 hours. Analysis of the sugars gave questionable data (Table 8). The specific activity of maltose and fructose remain very low because of little radioactive incorporation through all treatments. However, the specific activity of the two prevalent sugars, glucose and trehalose, varies considerably. It was assumed that this variability is a result of the different treatments. The concentrations of glucose and trehalose remain relatively constant in the controls and vary in the cold treated groups. Trehalose levels remain relatively constant with the exception of 24 hour cold group. This supports Wyatt's (1967) work which demonstrated that trehalose levels are one of the most stable carbohydrate pools in the Insecta, in contrast to glucose or fructose. Interestingly there appears to be a drop in the glucose

levels while trehalose remains constant. According to Wyatt (1967) this might be expected if one considers the stress of starvation analogous to that of cold stress. The stressed organism uses the glycogen and glucose stores before it uses the trehalose reserves. With the onset of a cryotaxic response, glucose may be mobilized. If that is true, a number of metabolic pathways include glucose as an intermediate. Thus as other unlabeled carbohydrates are converted to glucose the glucose pool is being diluted, resulting in a drop in the specific activity of glucose (Figure 1).

Comparison of the specific activity of glycogen fractions (Table 9) from warm and cold treatments show that in the cold there is an initial drop in the specific activity. However, it gradually gains activity in time. It is significant to note that there is great variation in the glycogen levels. Ants weighing approximately the same, and exposed to what was thought the same conditions, showed variations of 30-40% in tneir glycogen levels. Thus it is clear there are great variences in the physiological state of the ants which poses problems for future experimentation of this sort. A possible approach which might alleviate this problem in the future would be to culture the ants under more rigid conditions, or to work with larger numbers. By using larger samples, variation would be minimized.

Integrating the results of this study and the results of other workers, it is apparent that cold hardiness and the accumulation of glycerol as a result of a cryotaxic response of the organism is a much more involved process than originally thought. This study has laid a foundation from which specific experiments may be designed

to gain a better understanding of this cold adaption phenomenon. In light of the recent discovery of Van Handel (1970) that a secretion from the medial neurosecretory cells suppresses glycogen synthesis, it would be valuable to see if this also has a controlling effect on glycerol synthesis, in that glycogen metabolism appears intimately involved with glycerol synthesis.

A second approach to exploring this problem of glycerol synthesis is to inject the ants with position labeled glucose. As shown in Figure 5, the enzyme, aldolase catalyzes the cleavage of fructose 1,6-diphosate between C-3 and C-4. Carbons 1 thru 3 always yield dihydroxyacetone phosphate (DHAP), and C-4 thru 6 always yield glyceraldehyde-3-phosphate (G-3-P). Following injection of glucose-1-^{14}C and a period of cold stress, the glycerol could be isolated. If it were labeled, one would assume glucose to be a precursor; if not labeled, this would strongly indicate that glycerol does not arise directly from glucose. As shown in Figure 5, the enzyme triose isomerase catalyzes the reaction DHAP to G-3-P. If assays were run for both warm and cold extracts one could test for the cold inactivation of this enzyme, which would favor a buildup of glycerol as a result of a shift in equilibrium.

CONCLUSIONS

As a result of the experiments conducted, the following conclusions have been made:

1. The following Ichneumons accumulate glycerol when cold stressed:

Aoplus confirmatus Cress.

Hoplismenus m. moralus Say.

Ichneumon centrator Say.

Ichneumon fuscifrons Cress.

Ichneumon gradisops Hein.

Ichneumon heterocampae Cush.

Orgichneumon calcatorius Thun.

Ichneumon sp. (males)

2. In the Ichneumonidae, the ability to overwinter as free adults is associated with the accumulations of glycerol at low temperatures.

3. In *Camponotus pennsylvanicus* (L.) glycerol levels were found to be uniform in all body regions.

4. All four castes of ants accumulate glycerol when cold stressed.

5. In the ants, there is a linear glycerol accumulation with time.

6. Ants do not contain any appreciable alcohol dehydrogenase.

7. α-glycerophosphate dehydrogenase is not activated in the cold.

8. Total glycogen levels decrease when the organism is cold stressed.

9. Total glucose levels decrease when the ants are cold stressed.

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APPENDIX

TABLES AND FIGURES

FIGURE 1.--A Diagram of Glycolysis Showing the Pathway of Glycerol and Sorbitol Synthesis

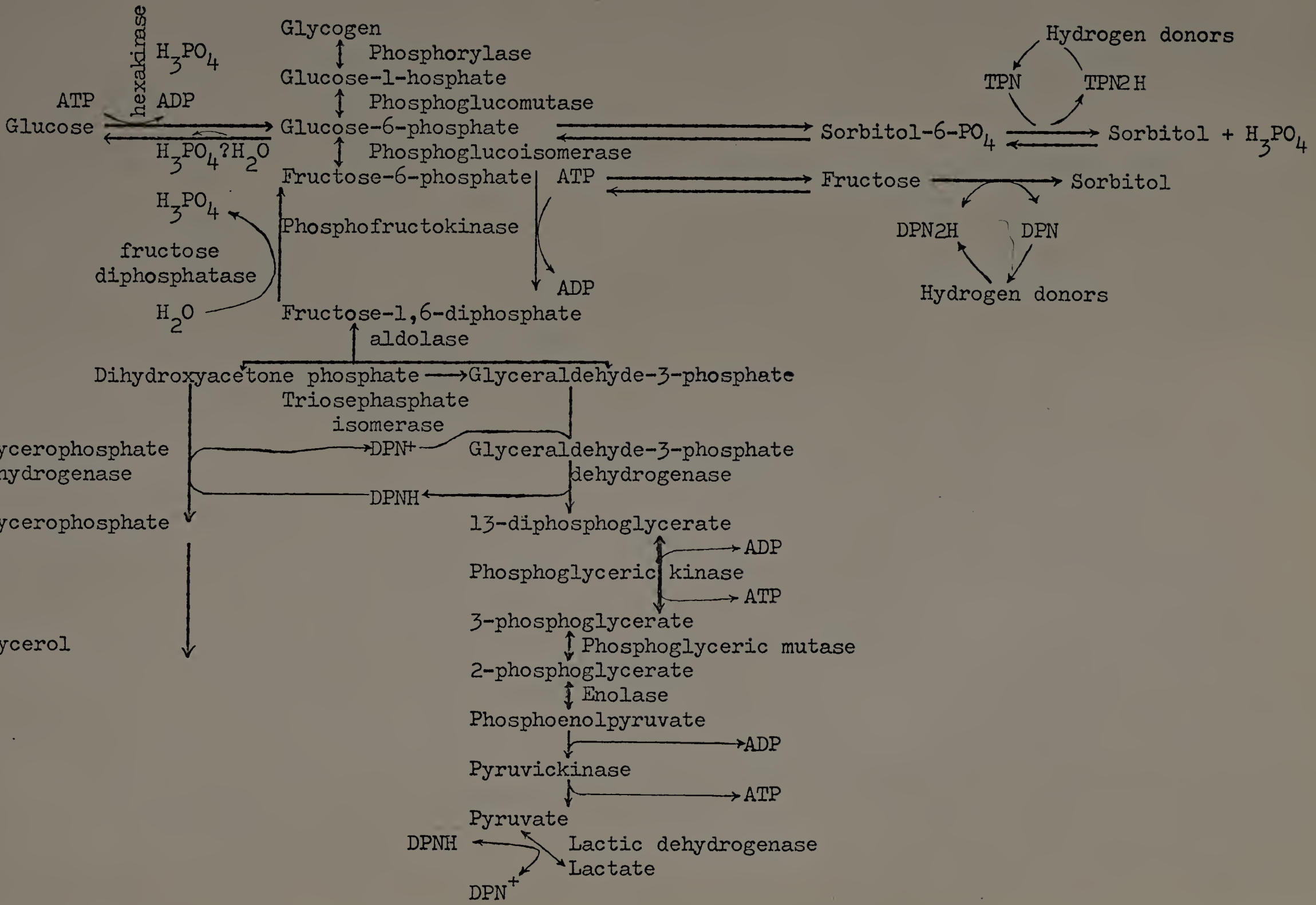

TABLE 1.--Glycerol Content of Hibernating and Non Hibernating Species of Ichneumon Wasps

Hibernators		Glycerol % Fresh Body wt.	
1.	Aoplus confirmatus Cress.	.07	
2.	Hoplismenus m. moralus Say.	.12	.46*
3.	Ichneumon centrator Say.	.08	.35*
4.	Ichneumon fuscifrons Cress.	.12	
5.	Ichneumon grandisops Hein.	.03	
6.	Ichneumon heterocampae Cush.	.02	
7.	Orgichneumon calcatorius Thun.	.05	
8.	Ichneumon sp.		.2**
Non Hibernators			
9.	Megarhyssa macrurus Linn.	0	
10.	Megarhyssa atrata Fab.	0	
11.	Habronyx sp.	0	
12.	Euceros sp.	0	
13.	Arotes sp.	0	

* -1° for 1 month

** 0° for 1 week (male)

FIGURE 2.--Plot of time vs. glycerol concentration in *C. pennsylvanicus*

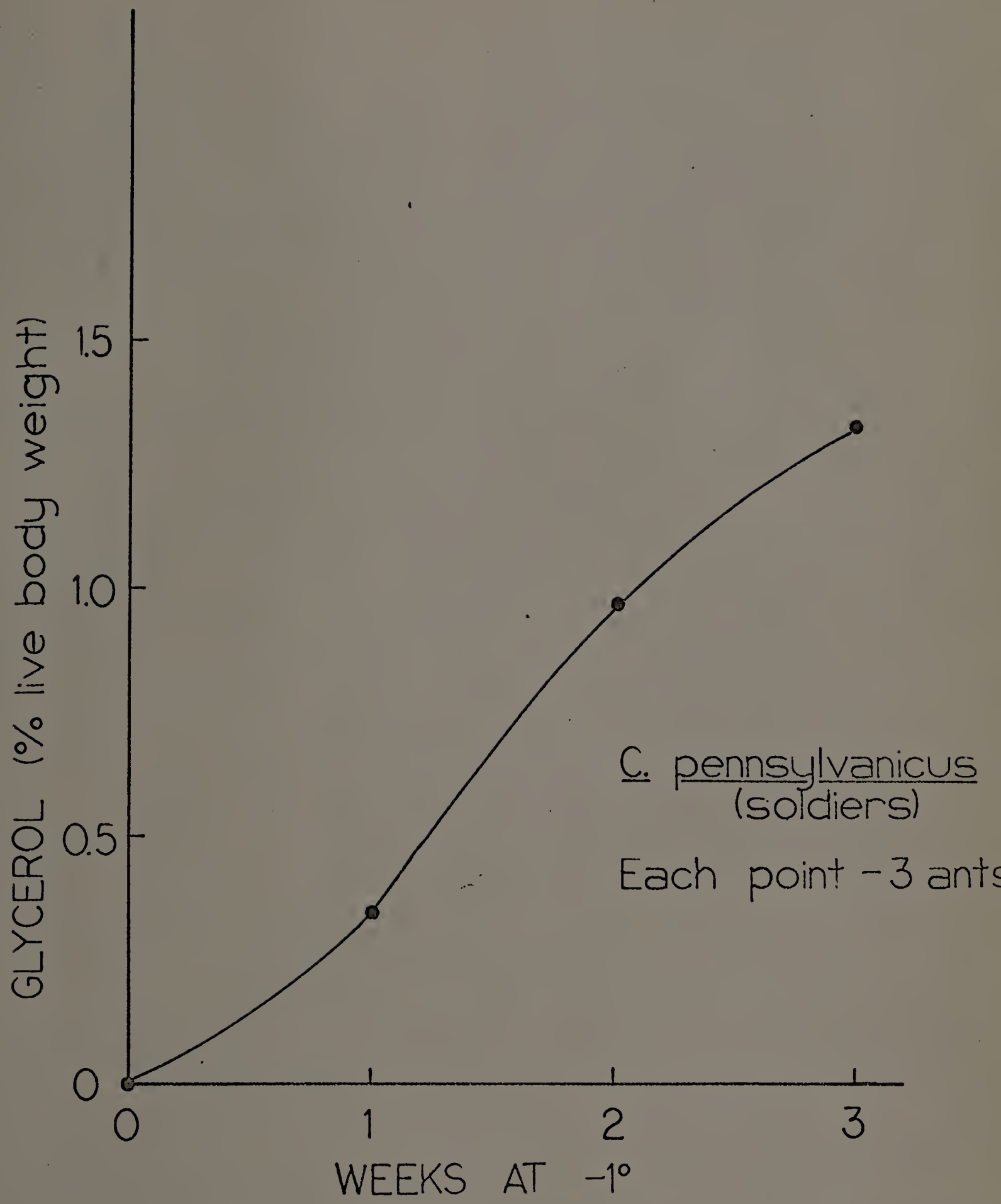
GLYCEROL (% live body weight)
1.5
1.0
0.5
0
0
1
2
3
WEEKS AT -1°
C. pennsylvanicus
(soldiers)
Each point - 3 ants

FIGURE 3.--Plot of time vs. glycerol concentration in *C. pennsylvanicus*

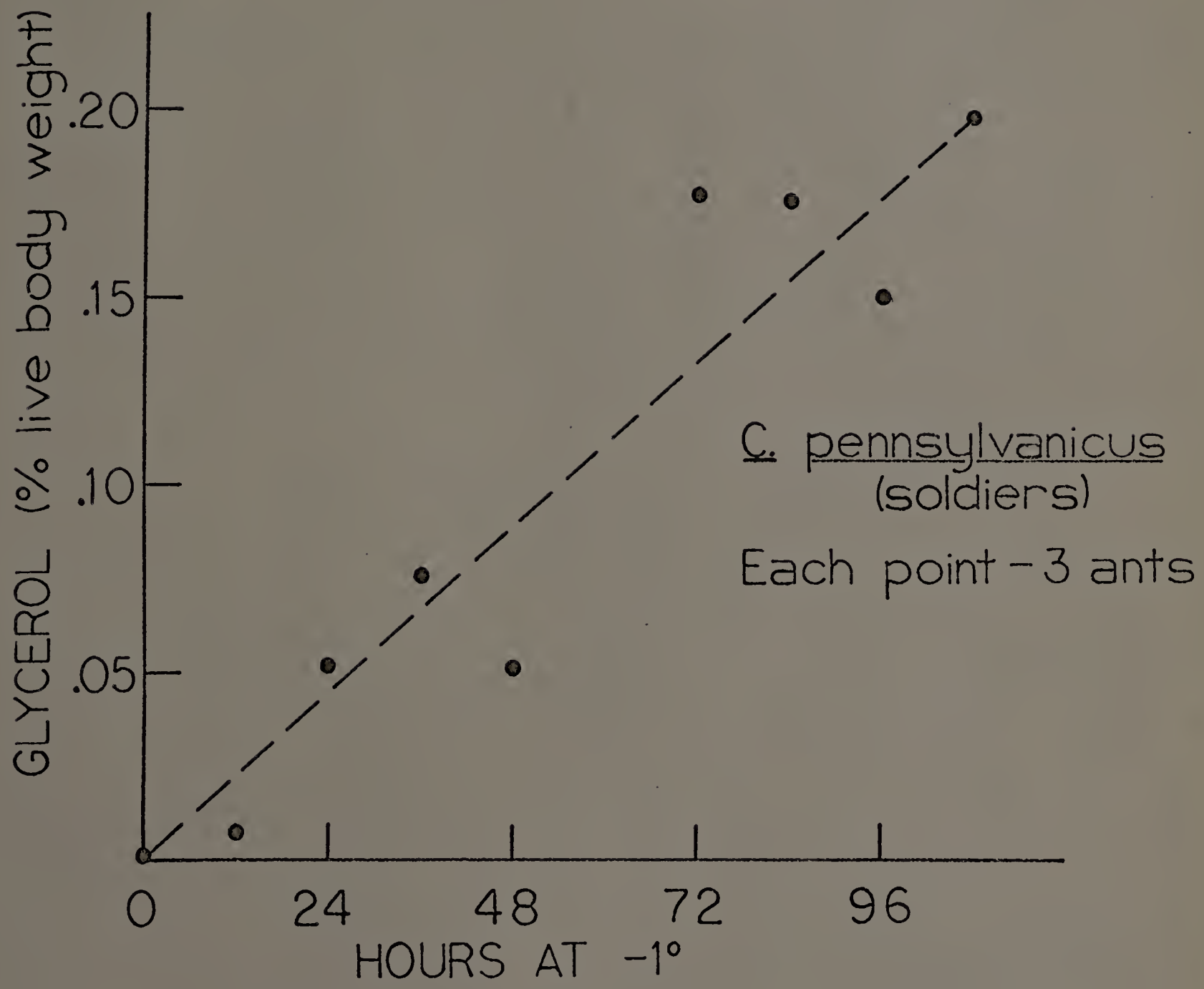
GLYCEROL (% live body weight)
.20
.15
.10
.05
0
24
48
72
96
HOURS AT -1°
C. pennsylvanicus
(soldiers)
Each point - 3 ants

TABLE 2.--Glycerol Concentration in Various Body Regions
(Female Reproductives)

Region	Number Used	Fresh Weight (g.)	% Glycerol* (Average)
Heads	6	0.0492	1.30
Thoraces	6	0.0512	1.38
Abdomens	6	0.1003	1.09

*Based on fresh body weight.

TABLE 3.--Glycerol Accumulation in Different
Castes of Camponotus pennsylvanicus

Caste	Number Used	Fresh Weight (g.)	% Glycerol* (Average)
Female Reproductives	2	0.2552	2.5
Soldiers	4	0.1966	1.6
Workers	4	0.0539	2.0

Insects were analyzed for glycerol by gas chromatography after several months hibernation at -1°C.

*Based on fresh body weight.

FIGURE 4.--Plot of time vs. glycerol concentration in *C. pennsylvanicus*

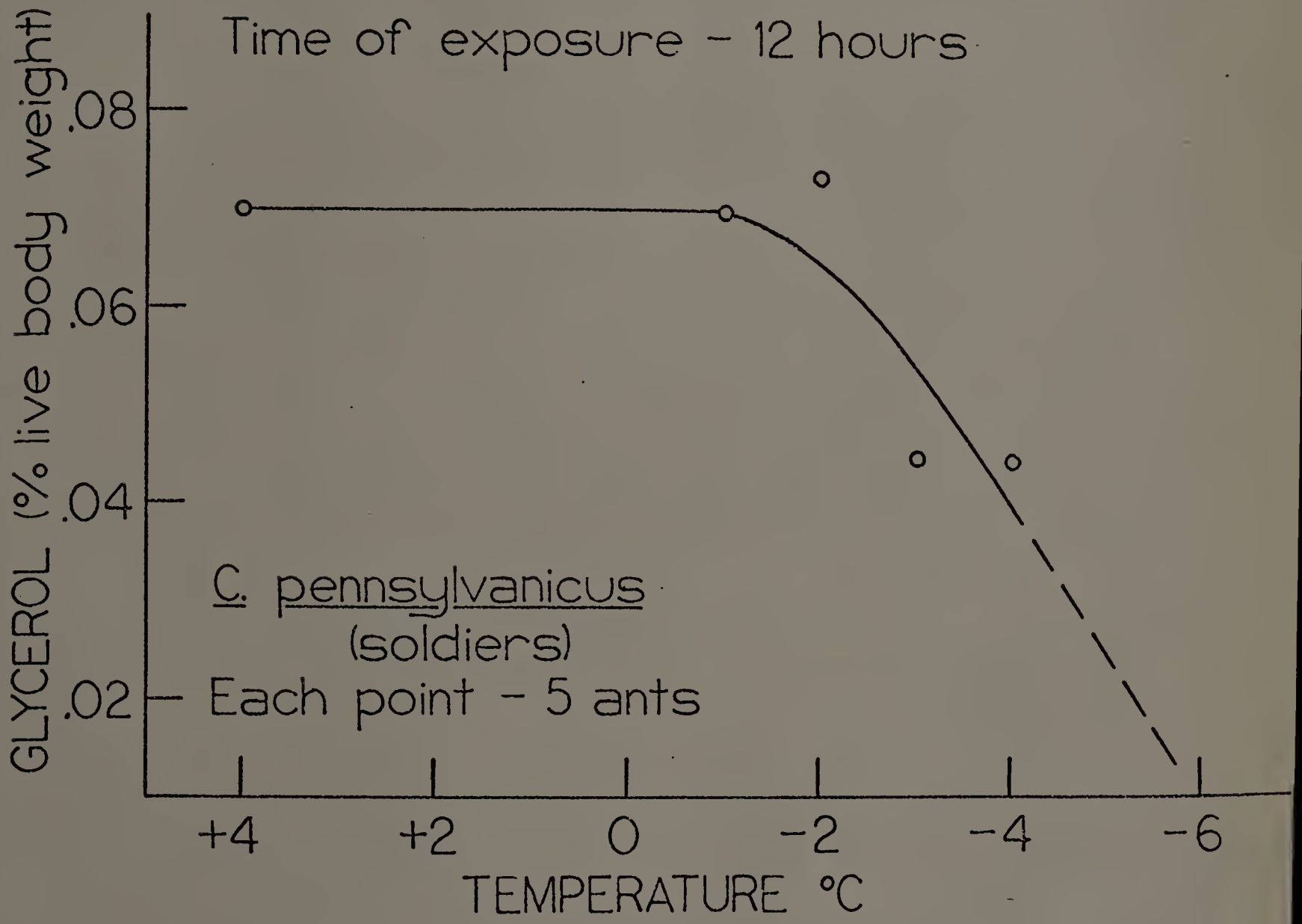
Time of exposure - 12 hours
GLYCEROL (% live body weight)
.08
.06
.04
.02
C. pennsylvanicus
(soldiers)
Each point - 5 ants
+4
+2
0
-2
-4
-6
TEMPERATURE °C

TABLE 4.--Glycogen Levels in Hibernating and Non Hibernating Ants C. pennsylvanicus

Experiment	Caste	Environmental Temperature		Glycogen* Micrograms / Milligrams Fresh Wt.
1	Female Reproductives	+22°		19
	Female Reproductives	-2°	(6 weeks)	14
2	Soldiers	+22°		20
	Soldiers	-2°	(3 weeks)	8
	Soldiers	-2°	(6 weeks)	0
3	Soldiers	-2°	(6 weeks)	0
	Workers	-2°	(6 weeks)	0
	Female Reproductives	-2°	(6 weeks)	11

* Average value of sample from 3 ants in each caste

FIGURE 5.--Possible Enzymatic Rx which might account for Glycerol Accumulation.

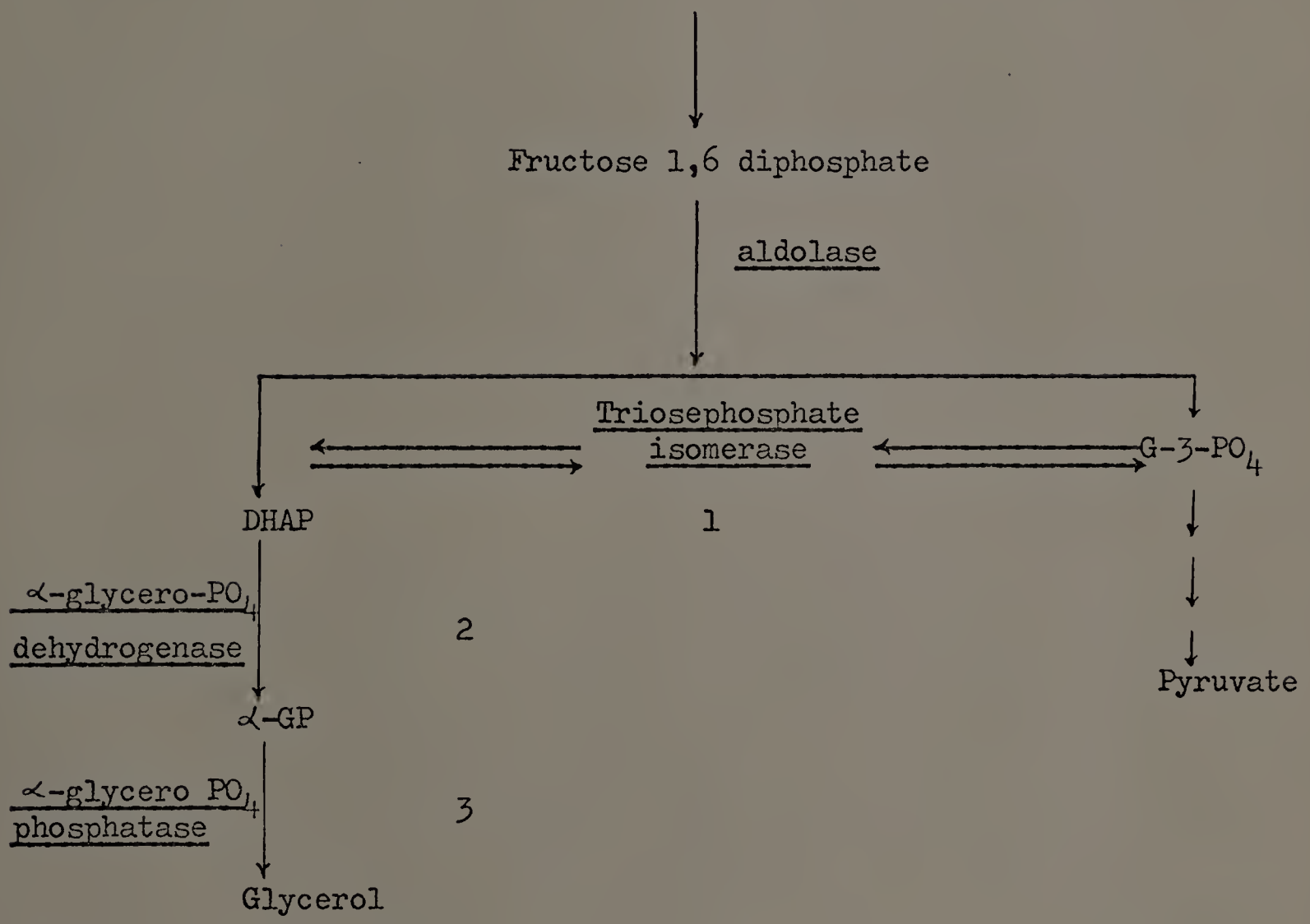

TABLE 5.--^{14}C-Glycerol Accumulation in Female Reproductive

Treatment, hours in cold	Fresh Weight (g.)	Total # Counts (CPM)	CPM / mg. Ant
8	0.2672	30,600	114
24	0.3539	33,200	94
48	0.4520	23,800	52

TABLE 6.--Radioactivity in ^{14}C-Triglyceride Glycerol in Coldstressed Female Reproductives

Treatment, hours in cold	Fresh Weight (g.)	Total # Counts (CPM)	CPM / mg. Ant
8	0.2672	1,600	6
24	0.3539	2,200	6
48	0.4520	2,100	4.5

TABLE 7.--^{14}C-Glycogen Accumulation in Female Reproductives

Treatment, hours in cold	Fresh Weight (g.)	Total Glycogen (mg.)	Total # Counts CPM
8	0.2672	3.1	5,550
24	0.3539	3.7	840
48	0.4520	13.2	1,400

TABLE 8.--^{14}C-Levels in Glucose and Trehalose

Treatments	Fresh Weight (g.)	Total Glucose (mg.)	Total # Counts (CPM)	Specific Activity (CPM/mg.)
12 hr. cold	0.4059	3.78	72,400	19,000
24 hr. cold	0.4130	1.62	26,800	16,750
28 hr. cold	0.4108	1.53	22,800	15,000
24 hr. warm	0.3936	3.78	33,400	8,750
48 hr. warm	0.3871	3.78	26,500	6,950

Treatments	Total Trehalose (mg.)	Total # Counts (CPM)	Specific Activity (CPM/mg.)
12 hr. cold	0.55	38,700	70,000
24 hr. cold	1.28	53,400	41,000
48 hr. cold	0.81	8,000	10,000
24 hr. warm	0.62	26,000	42,000
48 hr. warm	0.69	22,600	33,000

TABLE 9.--^{14}C-glycogen Accumulation

Treatment	Fresh Weight (g.)	Total Glycogen (mg.)	Total # Counts (CPM)	Specific Activity (CPM/mg.)
12 hr. cold	0.4059	28.1	44,640	1,590
24 hr. cold	0.4130	12.9	20,940	1,620
48 hr. cold	0.4108	13.3	22,860	1,590
24 hr. warm	0.3936	17.7	45,900	2,600
48 hr. warm	0.3871	25.1	69,000	2,750